Utilization of the Nitrate Reductase Enzymatic Pathway to Reduce Enteric Pathogens in Chickens

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ABSTRACT Previous reports have shown that some bacteria, including *Salmonella*, use a dissimilatory nitrate reductase enzyme pathway (NREP) in anaerobic environments. This enzyme reduces nitrate to nitrite and has been shown to cometabolize chlorate to cytotoxic chlorite. The present investigations were performed to evaluate the susceptibility of a competitive exclusion culture (CE) to the experimental chlorate product (ECP). A commercially available CE product was evaluated for its nitrate reductase activity and therefore its chlorate sensitivity. Individual isolates (in triplicate) were cultured in 10 mL of Viande Levure broth containing 5 m*M* sodium nitrate or 10 m*M* sodium chlorate. Bacterial growth (optical density at 625 nm) was measured and 1-mL aliquots were removed concurrently for colorimetric determination of nitrate content at 0, 3, 6, and 24 h. Of the 15 different facultative strains, 11 had slight NREP utilization, 3 had moderate NREP utilization, and the remainder were NREP negative (with slight and moderate NREP utilization: >0.1 to <1.0 m*M* and >1.0 m*M* nitrate used within 6 h, respectively). Of the obligate anaerobes evaluated, 3 had slight NREP utilization and the remainder were NREP negative. In vivo studies utilizing both products (CE and ECP) in a horizontal transmission challenge model (seeders + contacts) showed significant reductions in *Salmonella* from 5.37 to 1.76 $\log_{10}$ cfu/g and 3.94 to 0.07 $\log_{10}$ cfu/g, respectively. The combined effect of the CE culture and an ECP are effective in killing these foodborne pathogens.

(*Key words*: chicken, chlorate, competitive exclusion, nitrate reductase)

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INTRODUCTION

Foodborne illness continues to be a serious problem in the United States. In 2003, there were more than 1.3 million cases of Salmonellosis resulting from foodborne illness with an estimated cost of 2.8 billion dollars in the United States (Economic Research Service, 2003). In the commercial poultry industry, *Salmonella* is a serious problem, because it has been identified in essentially all aspects of poultry production (Jones et al., 1991). The significance of the problem cannot be truly understood until consideration is given to current production statistics for poultry in the United States. United States production statistics reveal that more than 8.5 billion broiler chickens and 274 million turkeys were processed in 2003 (USDA, 2004). State and federal regulatory agencies have mandated that poultry producers comply with regulations to reduce the overall incidence of *Salmonella* in poultry and poultry products.

To reduce the amount of *Salmonella* coming into processing plants, which subsequently contaminates processed carcasses, poultry producers have at their disposal several new intervention strategies. One preharvest intervention strategy that is being evaluated in our laboratory for pathogen reduction is a chlorate ion based product that uses the respiratory nitrate reductase enzymatic pathway (NREP). The NREP is part of a nitrate respiration process in some enteric and sulfate-reducing bacteria; this pathway uses nitrate as a terminal electron acceptor during anaerobic metabolism (Richardson, 2001). The NREP cometabolically reduces chlorate to a cytotoxic chlorite ion; increased levels of this ion become lethal to bacteria (Stewart, 1988). Utilization of the chlorate ion by NREP has been shown to reduce enteric pathogens in chickens, sheep, beef cattle, and pigs (Anderson et al., 2001; 2002; Byrd et al., 2003; Edrington et al., 2003).

To gain a better understanding of the microflora affected by this experimental chlorate product (ECP), a competitive exclusion (CE) culture from chicken was eval-

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Abbreviation Key: CE = competitive exclusion culture; BGA = brilliant green agar; ECP = experimental chlorate product; NREP = nitrate reductase enzymatic pathway.

uated. Competitive exclusion products are used to establish a protective microflora in the gastrointestinal tract of neonatal poultry. Evaluating the individual CE bacteria with ECP would help determine the sensitivity of the healthy microflora to this product in the gastrointestinal tract. Many of the pathogenic bacteria in the family enterobacteriaceae such as *Salmonella* and *Escherichia coli* possess the NREP. The objectives of the present investigation were to evaluate the effects of the ECP on the CE culture and to evaluate the efficacy of the ECP in the reduction of *Salmonella* in broilers. The results of this study may help determine the compatibility of these products on gastrointestinal health, and is an important process in finding new technologies for the reduction of enteric pathogens.

MATERIALS AND METHODS

Experiment 1

In experiment 1, the CE culture Preempt[2] was evaluated to determine the sensitivity of the individual bacteria to an ECP and to measure the use of sodium nitrate. Each bacterial isolate tested was grown with and without 5 m*M* sodium nitrate[3] or sodium chlorate.[4] To start the initial bacterial isolates (previously isolated in our laboratory), 100 μL of individual stock culture was removed and placed in 10 mL of tryptic soy broth[5] and grown in anaerobic conditions for 48 h at 37°C (Corrier et al., 1995). A 100-μL aliquot was removed from the started cultures and placed in tryptic soy broth for 48 h at 37°C, in triplicate. A 1-mL sample of the culture from individual isolates was obtained at 0, 3, 6, 24, and 48 h. The optical density reading of the culture was recorded at each time in a spectrophotometer and absorption was measured at 625 nm. This reading was used to determine the inhibition of growth of the bacteria. A previously described colorimetric assay was used to determine the use of nitrate (Cataldo et al., 1975).

Experiment 2

For experiment 2, Cobb × Ross broiler chicks were obtained from a local commercial hatchery on day of hatch. All chicks were placed in rearing pens at appropriate rearing temperature on clean pine shavings litter material. All chicks were provided water and a corn-soy based diet that met or exceeded NRC guidelines (1994) for ad libitum consumption. In this experiment, the objective was to evaluate the CE culture and the ECP in the reduction of a foodborne pathogen. The experiment contained 4 treatment groups: 1) negative control, 2) CE culture, 3) ECP, and 4) a combination of the CE and ECP. On d 1, 25 chicks from each group were challenged with 10^8 to 10^9 cfu of *Salmonella* Typhimurium resistant to novobiocin[4] and nalidixic acid[4] (seeders) and placed in the pen with the remaining unchallenged chicks (contacts). One hour later, a reconstituted CE product was administered by oral gavage (0.25 mL) to groups 2 and 4. After an additional hour, chicks in groups 3 and 4 were provided water that contained ECP (1× ECP is equivalent to a 15 m*M* chlorate ion concentration) to groups 3 and 4 for 4 d. Chicks in groups 1 and 2 were provided distilled water for 4 d. After this period, all chicks were provided free access to water until the end of the experiment. On d 3, approximately 48 h postCE treatment, 10 chicks in each group were randomly selected and euthanized by cervical dislocation. The concentration of propionic acid and total volatile fatty acids (acetic + propionic + butyric + isobutyric + valeric + isovaleric) in the cecal contents were determined by gas liquid chromatography as described previously (Corrier et al., 1993).

Recovery of Salmonella

On d 10, the remaining seeder and contact chicks were killed by cervical dislocation and evaluated for *Salmonella* colonization. An individual cecum was removed and 0.25 g of cecal contents was placed into a 6-mL snap cap polypropylene tube containing 2.25 mL of Butterfield's solution. Serial dilutions of each sample were performed using 0.5 mL of the sample diluted in 4.5 mL of Butterfield's solution for a final concentration of 10, 100, and 1,000 cfu/mL. One hundred microliters from each dilution tube was placed onto a brilliant green agar[5] (BGA) plate containing 25 μg/mL of novobiocin and 20 μg/mL of nalidixic acid and spread plated using a bacterial cell spreader. All of the plates were incubated for 24 h at 41°C, and the number of *Salmonella* was determined and expressed as $\log_{10}$ cfu/g of cecal contents. Cecal contents that were negative at a 100-fold dilution on BGA plates but positive at a 10-fold dilution were assigned 1.50 $\log_{10}$ cfu/g of cecal contents (Corrier et al., 1993, 1995)

Statistical Analysis

Differences in propionate levels in the cecal contents of treated chickens were evaluated using the GLM procedure for one-way ANOVA (SAS Institute, 1996). Statistically different means ($P \leq 0.05$) were further separated using Duncan's Multiple Range Test (SAS Institute, 1996). The χ^2 test of independence was used to compare *Salmonella* Typhimurium incidence data following bacterial enrichment of cecal tonsils (Ott, 1993).

RESULTS AND DISCUSSION

The results from experiment 1 indicate how the ECP affected the CE culture in vitro. Of the 15 different facultative strains, 11 had slight NREP use, 3 had moderate NREP use, and the remainder were NREP negative (with slight and moderate use: ≥0.1 to ≤1.0 m*M* and ≥1.0 m*M*

[2]MS BioScience, Dundee, IL.
[3]Mallinckrodt Baker Inc., Paris, KY.
[4]Sigma Aldrich Co., St. Louis, MO.
[5]Becton Dickinson and Co., Sparks, MD.

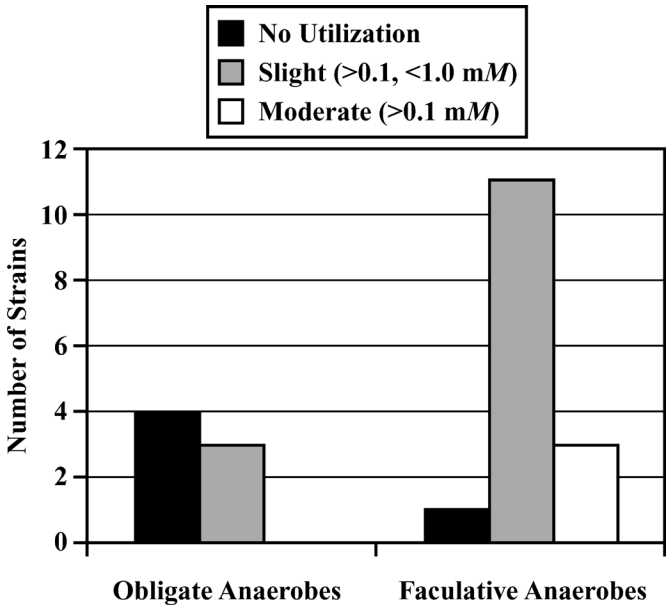

FIGURE 1. Nitrate (5 mM) utilization of obligate and facultative anaerobes in a commercial competitive exclusion culture.

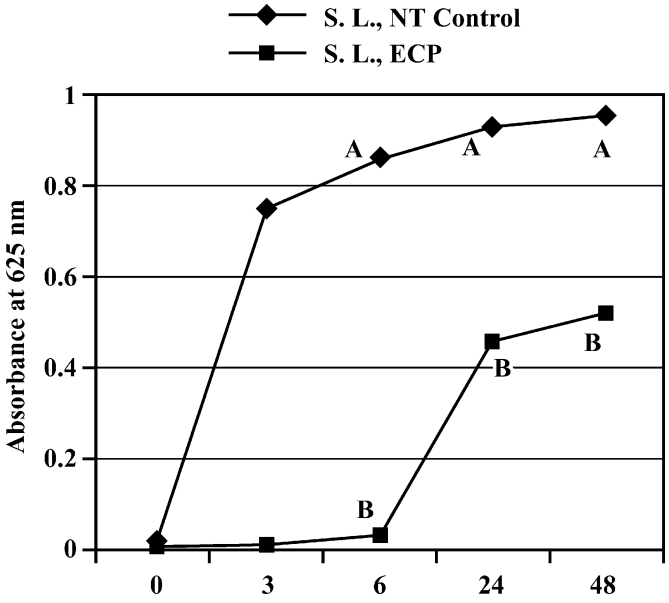

FIGURE 3. Utilization of the nitrate reductase pathway: a comparison of *Serratia liquefaciens (S. L.)* grown in the presence of nitrate (NT) or an experimental chlorate compound (ECP) for 0, 3, 6, 24, and 48 h, *ECP is a 15 mM chlorate ion equivalent. [A,B]Mean values with no common superscripts differ significantly ($P \leq 0.05$).

nitrate used within 6 h, respectively). Of the obligate anaerobes evaluated, 3 had slight use, and the remaining were NREP negative (Figure 1). Of the total bacteria that were NREP positive, 50% were ECP sensitive, as indicated by marked inhibition of growth during the 48-h incubation period. *Enterococcus faecalis* had a slight NREP use and had impaired growth when the ECP was present (Figure 2). *Serratia liquefaciens* had a moderate use of nitrate and had impaired growth when the ECP was present (Figure 3). *Enterococcus faecalis* and *S. liquefaciens* were selected as representatives of both slight and moderate use of the NREP pathway which cometabolically reduces the chlorate ion to cytotoxic chlorite. Although several of the bacterial populations are ECP sensitive, ECP does not eliminate all the bacterial constituents of this CE product.

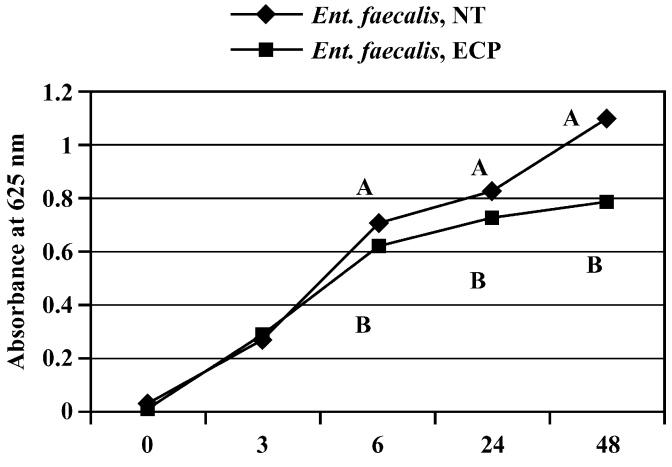

FIGURE 2. Utilization of the nitrate reductase pathway: a comparison of *Enterococcus faecalis* grown in the presence of nitrate (NT) or an experimental chlorate compound (ECP) for 0, 3, 6, 24, and 48 h, ECP is a 15 mM chlorate ion equivalent. [A,B]Mean values with no common superscripts differ significantly ($P \leq 0.05$).

In experiment 2, broiler chicks provided ECP in the drinking water in combination with a CE product had significant reductions in the number of *Salmonella* recovered and in overall incidence compared with the controls (Table 1). *Salmonella*-challenged (seeders) or unchallenged (contacts) chicks had significantly lower numbers of cecal *Salmonella* cfu recovered and lower incidence of *Salmonella* when compared with the controls. Experimental chlorate product and CE treatment groups had significantly lower numbers of *Salmonella* cfu recovered from the ceca and the incidence detected in the ceca as compared with the controls. Furthermore, the combination of ECP and CE was not significantly different from the ECP alone with the exception of nonchallenged chicks provided ECP alone. Experimental chlorate product alone or in combination with CE provided to broiler chicks 3 d before sampling did not significantly affect the cecal volatile fatty acid concentrations compared with the controls or the CE alone group (data not shown). The data in the present study suggest that ECP provided in the drinking water during the first 4 d of life may reduce the incidence and the population of *Salmonella* in newly hatched chicks. Furthermore, data suggest that ECP does not adversely affect the microbial population naturally found in the gastrointestinal tract.

These results agree with recent studies in our laboratory that demonstrated that chlorate supplementation effectively decreased *E. coli* O157:H7 in cattle and pigs before harvest (Anderson et al., 2001; Callaway et al., 2002). Studies demonstrated that chlorate significantly reduced *E. coli* O157:H7 and *Salmonella* Typhimurium

TABLE 1. Effect of a competitive exclusion culture (CE) and an experimental chlorate compound (ECP)[1] provided in the drinking water during the first 4 d on *Salmonella* Typhimurium cecal colonization in broiler chicks (2 trials)

	Salmonella-challenged (seeders)		Unchallenged (contacts)	
Treatment	Log_{10} cfu/g of cecal contents[2]	*Salmonella* positive chicks per total chicks (%)	Log_{10} cfu/g of cecal contents	*Salmonella* positive chicks per total chicks (%)
Control	5.37 ± 0.98[a]	40/40 (100)[a]	3.94 ± 2.15[a]	33/39 (84.6)[a]
CE-gavage	4.21 ± 2.05[b]	35/40 (87.5)[b]	1.31 ± 2.08[b]	13/39 (33)[b]
ECP	1.71 ± 2.31[c]	16/39 (41)[c]	0.42 ± 0.97[c]	7/40 (15)[b]
CE + ECP	1.76 ± 2.37[c]	15/40 (37.5)[c]	0.07 ± 0.45[c]	1/40 (2.5)[c]

[a-c]Mean values within the same column with no common superscripts differ significantly ($P \leq 0$ .05).

[1]ECP is equivalent to a 15 m*M* chlorate ion concentration.

[2]Mean ± SD.

DT104 in gastrointestinal contents and did not significantly alter normal total culturable anaerobic bacteria counts. These results suggest that chlorate supplementation may be a viable strategy to reduce foodborne pathogens that possess NREP. Previously, Davies and Wray (1996) suggested that preharvest *Salmonella* control might be most effective when applied in the final period before harvesting. Because poultry have access to water during initial placement and the feed withdrawal period and ECP can be administered in the drinking water, ECP could have an impact on the pathogen load in a preharvest setting. The combined effects of the ECP with other products such as CE could provide a practical approach for reducing foodborne pathogens at initial placement and at entry into the processing plant. Government regulations (Pathogen Reduction Act) have caused a need for cost efficient approaches to reducing foodborne pathogens without dramatically altering present management techniques. The results of the present study suggest a possible method to reduce foodborne pathogens that can be incorporated into existing commercial management procedures.

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